SENSITIVE AND RAPID BLOOD AND TISSUE HPLC OXIME ASSAY AND PHARMACOKINETICS OF MMB-4 IN GUINEA PIGS AND AFRICAN GREEN MONKEYS

Gregory E. Garcia*, Harry Singh, Deborah Moorad-Doctor,
Ruthie H. Ratcliffe, Katie Wachtel, Andres Castillo, Richard K. Gordon
Walter Reed Army Institute of Research, Silver Spring, MD 20910
Todd M. Myers, John H. McDonough,
USA Medical Research Institute of Chemical Defense, APG, MD

ABSTRACT

We developed a rapid and sensitive assay for pyridinium oximes in plasma and tissues. Samples are prepared by acidification and then deproteinized by ultra-filtration. The oximes are measured during sample HPLC fractionation over a cation-exchange column with UV detection. The assay is suitable for analysis of mono- and di-pyridinium oximes, but was originally developed for the measurement of the oxime MMB-4. For both plasma and tissue sources, the LLOD was 0.0005 μg and the LOQ was 0.001 to 2.5 μg. The assay requires as little as 50 μL of whole blood or 30 μL of tissue homogenate. The assay was used for plasma pharmacokinetic studies from a single intramuscular injection of MMB-4 (dichloride or dimethylsulfonate salt) in nonhuman primate African green monkeys (AGM), and the plasma and tissues of the rodent guinea pig (GP). For the two species, both MMB-4 salts were pharmacokinetically equivalent. In AGM plasma, the C_{max} and the area under the curve (AUC) varied dose-dependently with a T_{max} of approximately 20 min and mean residence time (MRT) was approximately 92 minutes for all doses of both salts. In GP plasma and muscle tissues, the C_{max} and AUC also varied dose-dependently. The plasma T_{max} was about 34 to 42 min while the muscle tissue levels peaked at 5-20 min. The tissue concentrations were much lower than the plasma. The tissue levels peaked at 5-20 min depending on the tissue with a concentration ranking of diaphragm > heart > thigh muscle.

1. INTRODUCTION

Organophosphate (OP) nerve agents inhibit cholinesterases (ChE) by reaction with the ChE active-site serine. The phosphorylation is initially reversible; however, a loss of an R-group from the phosphate and the serine modification is essentially irreversible. The irreversible inactivation is known as 'aging'. However, a nucleophilic attack on the OP phosphorous can displace the OP off the ChE serine before aging has occurred and reactivate the enzyme. Oximes are reactivators of OP-ChEs as the oxime can provide the nucleophilic attack (1-3). Antidotal treatment in the United States (US) for OP poisoning has been the mono-pyridinium mono-oxime 2-pralidoxime chloride (2-PAM). 2-PAM is a good reactivator of ChEs inhibited with sarin and VX, but is not as effective for soman and tabun (4). The oxime HI-6 is more effective against sarin, soman, and VX but not tabun. Trimedoxime is more effective against tabun (5-8). HI-6 is the oxime used by some non-US NATO forces. To overcome these shortcomings, other oximes are needed that have broader specificity. This has led to the development of MMB-4; *1,1'-methylenebis{4-[(hydroxyimino)methyl]pyridinium} dichloride/disulfonate* salts (9). MMB-4 is a leading candidate to replace 2-PAM in the US, and we have developed quick and sensitive methods to determine MMB-4 levels in plasma and tissues and to evaluate its pharmacokinetics. These methods were also found to be easily adaptable for analysis of other mono and di-pyridinium oximes.

2. MATERIALS AND METHODS

2.1 Materials

Certified oximes: dichloride (DC) and dimethylsulfonate (DMS) salts of 1,1'methylenedbis{4-[(hydroxyimino)methyl]pyridinium} (MMB-4), 329 and 450 g/mol respectively; 1,1'trimethylenedbis{4-[(hydroxyimino)methyl]pyridinium} (TMB-4); 1-(2-hydroxyiminomethyl-1-pyridino)-3-(4-carbamoyl-1-pyridino)-2-oxapropane dimethylsulfonate (HI-6); 1-(4-aminocarbonylpyridininio)-3(2-hydroxyiminomethylpyridino)-propane dichloride monohydrate (ICD-0585, methylene bridge analog of HI-6); 1-[2'-(hydroxyimino)methyl]-3'-methyl-1'-imidazolyl-3-(4'-carbamoyl-1'-pyridinyl)propane dichloride (ICD-0692); and 3-(4-carbamoyl-1-pyridino)-1-(2,4-bis(hydroxyimino-methyl-1-pyridio)-2-oxapropane dimethylsulfonate (HLö7) were obtained from the Walter Reed Army Institute of Research Chemical Repository in powder form. The oximes were stored in a desiccator at -30°C. Pooled normal African green monkey and guinea pig plasma was obtained from Biomeda Corp. (Foster City, CA). Other chemicals were ACS grade and obtained from Sigma-Aldrich (Saint Louis, MO). The BCA protein assay kit was obtained from Pierce Biotechnology, Inc.

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(Rockford, IL). All solvents were HPLC grade and obtained from JT Baker, Ltd (Phillipsburg, NJ). Water was purified through a Milli-Q water purification system (Millipore, Inc., Billerica, MA) and was 18 mOhm.

2.2 Oxime Assay

The HPLC column used a weak-cation exchange PolyCAT A (2.1 x 100 mm, 5 μm, 300 Ang) inline with a matched Javelin guard cartridge (10 mm x 2.1 mm; 5 μm particle; 300 Ang.) both from PolyLC, Inc., Columbia, MD. The HPLC consisted of a Beckman 128 pump, 168 detector, and a Varian temperature-controlled autosampler at 4°C (± 4°C) controlled by Beckman 32Karat™ Ver. 5.0 software. MMB-4 was eluted using isocratic conditions at a flow of 0.3 mL/min with a retention time of ≤ 4 min. The HPLC solvent was 0.19 M ammonium acetate pH 4.5:44% acetonitrile. The ammonium acetate was filtered through a 0.45 micron filter prior to addition of the acetonitrile. Elution of MMB-4 was monitored at 300 nm, the absorbance maxima in the solvent (data not shown). TMB-4 was analyzed with a solvent composition of 0.21 M ammonium acetate pH 4.5/44% acetonitrile and monitored at 300 nm. Chromatography of 2-PAM was done with a solvent composition of 0.03 M ammonium acetate pH 4.5/44% acetonitrile and monitored at 296 nm. Sample MMB-4 plasma concentrations were determined from comparison to peak area standard curves of MMB-4 spiked matrix matched control (MMC, section 2.4) plasma; MMC was prepared by spiking unadulterated control AGM plasma (Bioreclamation, Inc., Hicksville, NY) with MMB-4. The MMC concentration range was 0.3, 0.03, 0.003 and 0.0003 μmolmL-1. The spiked MMCs were processed similarly as the samples.

The two salts of MMB-4 were prepared as 10 mg/mL solutions in 10 mM sodium phosphate, pH 7, filtered through 0.45 micron filters and stored at –80°C. There was no observable decomposition of the frozen oxime solutions over 12 months (data not shown). A molar extinction coefficient was determined from the absorbance maximum at 297 nm to be 34,851/cmM at pH 7, 10 mM sodium phosphate. Serial dilutions were prepared in the HPLC solvent.

2.3. Sample preparation

African green monkey and GP plasmas obtained from the pharmacokinetic study was stored at -80°C. To assay, the samples were thawed on ice and then clarified by centrifugation at 4°C for 5 min. Typically, 100 μL of plasma was used. For samples with high oxime concentrations, the samples were diluted with 0.9% NaCl, 50 mM phosphate, pH 7. They were then acidified with phosphoric acid to 3%, mixed, and again clarified by centrifugation. Next, the samples were processed by ultra-filtration (Vivaspin 500; 10,000 MWCO; Vivascience, AG, Germany) by centrifugation at 12,000 x g for 12 min at 4°C. The filtrate was collected into a clean microfuge tube for storage at -80°C or a HPLC sample vial for immediate analysis. Stored sample filtrates were thawed and then clarified by centrifugation at 4°C before analysis. A 10 μL injection volume was used.

GP muscle samples (diaphragm, heart, and thigh) were prepared by grinding to a powder under liquid N_2 with a mortar and pestle. The powder was stored at -80°C. For analysis, the frozen tissue powder was weighed and thawed in 3 volumes of ice-cold PBS. Homogenization was completed by 10 strokes each with loose and tight pestles of a Dounce homogenizer. The homogenate was clarified by centrifugation and an aliquot was taken for protein analysis. The sample was then acidified and processed in Vivaspin similar to the plasma samples described above. Precision was given as percent relative standard deviation (RSD) where RSD = (SD/mean)*100.

2.4 Matrix Matched Controls

A standard equation was derived from linear regression analysis of matrix matched control sets to more accurately quantify oxime detected in plasma samples and tissue homogenates. Pooled normal AGM and GP plasma and GP tissue homogenates from untreated animals were spiked with known amounts of MMB-4. The spiked plasma was then serially diluted with control plasma and incubated at 37°C for 30 min. Tissue homogenates were spiked, mixed, serially diluted with control homogenate, and processed immediately.

2.5 Pharmacokinetics of MMB-4 in Guinea Pig and African Green Monkeys

The doses of MMB-4 given to the guinea pigs were derived from the OP exposure oxime treatment regimen of the Swedish military. The Swedish military use the bis-pyridinium oxime HI-6 (Fig. 1 D) and three injectors to dose a 70 kg human with the HI-6 dichloride salt (57.9 μmol/kg for a total of 4060 μmol), where one injector contains 1353 μmol for 19.3 μmol/kg. The animals were given human dose equivalents of 19.3 and 58 μmol/kg, 1 x and 3 x dose, respectively. Injectable oxime solutions were prepared in PBS. The solutions were sterile filtered prior to use through 0.22 micron syringe filters. Twenty guinea pigs (n = 2 per time point) were injected intramuscular (i. m.) with oxime in the caudal thigh muscle. One group of animals (n = 2) were injected with the vehicle to serve as a negative control. Following oxime administration, 100 μL of blood was collected from the marginal ear vein at various time intervals. The blood was collected with a pipette tip treated with heparin and added to a collection tube containing ~1000 units of dried heparin. The blood was thoroughly mixed and the red blood cells removed by

centrifugation at 1,375 x g for 5 min at 4°C. The cell free plasma was collected, frozen on dry ice, and then stored at -80°C. At the designated time points the animals were euthanized by CO_2. Tissue samples were removed, frozen on dry ice, and then stored at -80°C.

Eight young adult male AGM (Chlorocebus aethiops) of Caribbean origin were used. Animals were placed individually in primate collar-restraint chairs and trained to be compliant during blood sampling and injections. A minimum of 14 days separated each injection. Blood samples were taken at 0 min (pre-injection baseline), 5, 10, 20, 30, 40, 80, 160, and 320 min following intramuscular injection of the test compound. Test compounds were MMB-4 diCl (molecular weight = 329.19 g) and MMB-4 DMS (molecular weight = 478.2 g) at molar-equivalent doses of 19.3, 58, and 116 µmolµ/kg. The volume of all injections was constant at 1 mL. The average animal weight was approximately 6.5 kg. The test compound was injected into the lateral thigh muscle. Signs of necrosis were examined at the injection site 24 and 48 h after injection. No signs of necrosis were observed. Whole blood (~0.8 mL) was collected from the saphenous vein into a 1 mL syringe (coated with 1000 Units/mL heparin). The blood was quickly processed in a microcentrifuge at 10k RPM for 3 min. The plasma was flash frozen in a dry ice and ethanol bath. The samples were stored at -80°C until processing as similarly described for GP.

Pharmacokinetic parameters were determined from the time course curve of plasma and tissue MMB-4 concentration: maximal concentration (C_{max}), time to reach the maximal concentration (T_{max}), elimination half-life ($T_{1/2}$), and area under the concentration time curve extrapolated to infinity (AUC) using Prism Software Ver. 4.03 (Graphpad Software, Inc., San Diego, CA).

This research was conducted under protocols approved by the United States Army Medical Research Institute of Chemical Defense (USAMRICD) and Walter Reed Army Institute of Research Animal Care and Use Committees in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals, and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, NRC, 1996.

2.6 Miscellaneous Procedures

Protein concentrations were determined by the micro BCA protein assay following manufactures instructions (Pierce Biotech., Inc.) using BSA as standard. The absorbance was determined using a Molecular Devices Spectra Max plus Model 384 plate reader at 562 nm.

3. RESULTS AND DISCUSSION

The FDA requires pharmacokinetic studies of a product – the new oxime MMB-4 - in blood and tissue of rodents and non-human primates to satisfy the "two-animal rule" for a product if efficacy can not be evaluated in humans. Therefore, we developed rapid and sensitive HPLC analytical procedures for MMB-4. Although our

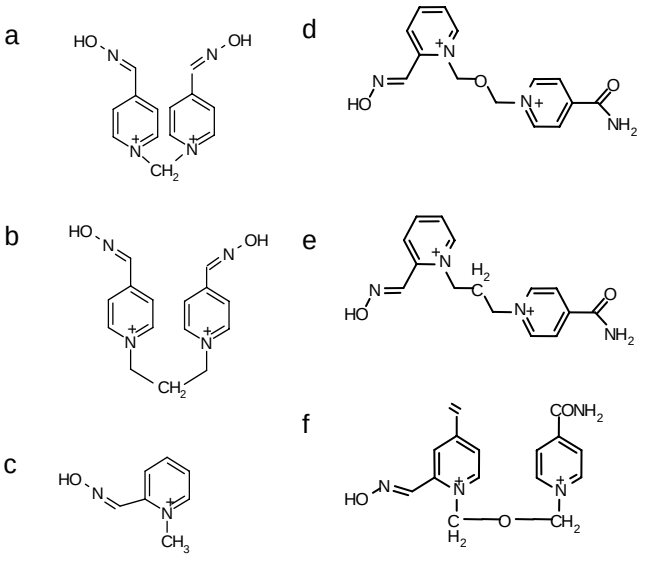

Fig. 1. Structures of Oximes: a. MMB-4; b. TMB-4; c. 2-PAM; d. HI-6; e. ICD-0585 (bridge analog of HI-6); f. HLö7.

work described here is for MMB-4, our assay is suitable for di-pyridinium oximes, including HI-6, ICD-0585, ICD-0692, HLö7, and also mono-pyridinium oximes such as 2-PAM (structures shown in Fig. 1) with chromatography examples shown in Fig. 2. Both the chromatography conditions and detection by UV absorbance are specific for the oxime, maximizing detection sensitivity, specificity, and quantitation. Under these conditions, the MMB-4 retention time (RT) is 3 to 4 min with a total run time of 6 min. To measure an oxime in tissue, some additional processing was required. The precision of the HPLC intra-day variability was determined using duplicate injections of 0.5, 5.0, 50.0 and 500 ng of MMB-4 injected twice daily. The relative standard deviation (RSD) ranged from 1.0% to 2.6%. Inter-day variability was determined for the same concentrations over three days and yielded a 6.9% overall mean and a range of 4.5% to 12.5%, indicating the reproducibility was very good. Recovery of MMB-4 from plasma was determined from oxime spiked control plasma; the spiked samples were mixed, incubated for 30 min at 37°C, and then acidified as described for plasma from animals. Plasma spiked with 0.1, 1.0, 10, 50, and 100 µg/mL MMB-4 yielded recoveries ranging between 86 to 98%. The recoveries of other oximes were: 79-96%

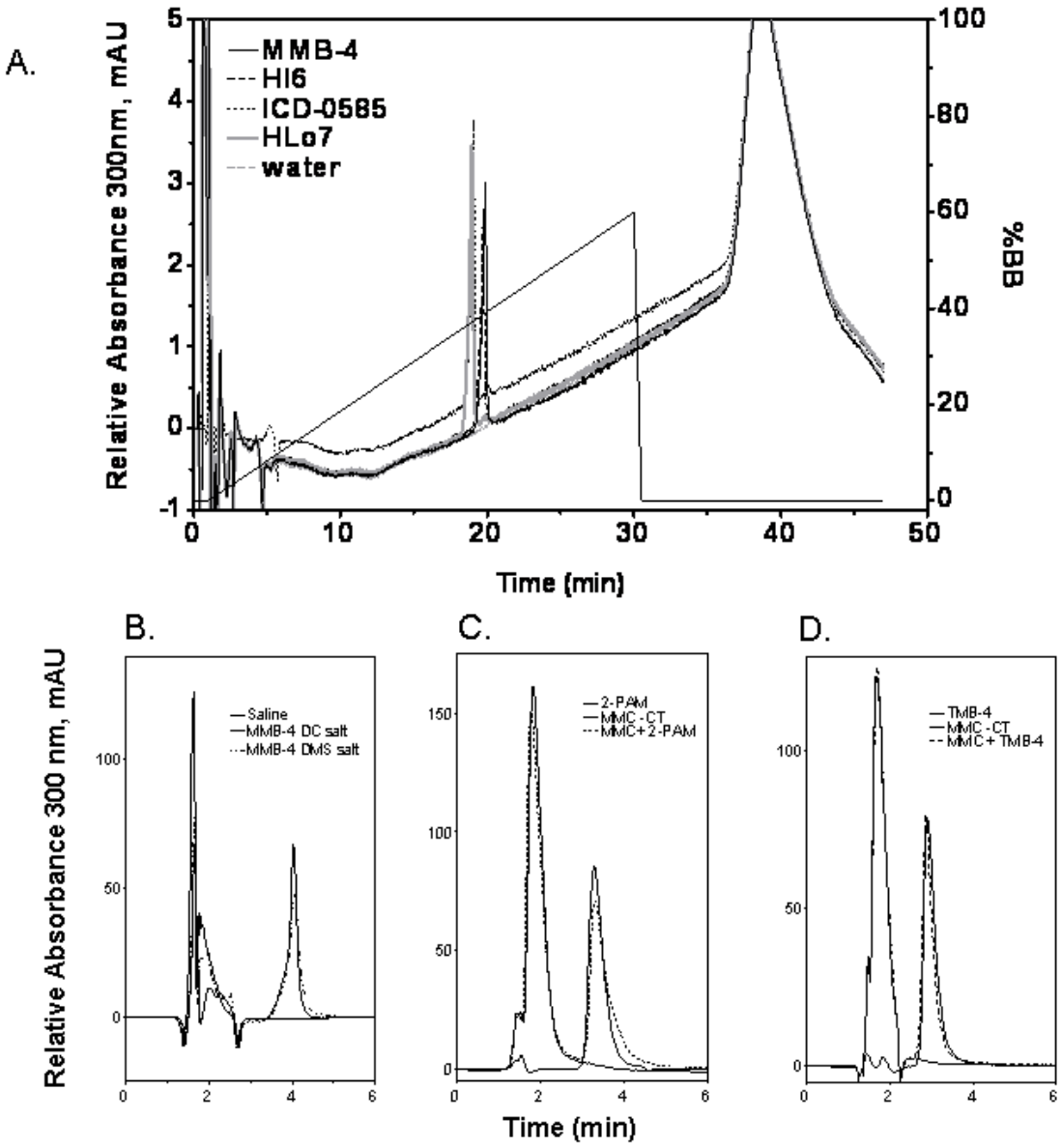

Fig. 2. Analysis of oximes by cation-exchange HPLC. A, gradient elution of indicated oximes from 10 μL injections. Oximes prepared in water at 1 μg/mL. B, analysis of plasma from guinea pigs treated with19.3 μmol/kg MMB-4, 5 min post–dosing, blood was removed and processed as described in Methods. Isocratic elution with 0.19 M ammonium acetate pH 4.5:44% acetonitrile of 10 μL sample injections. C and D, analysis of 2-PAM and TMB-4 spiked control guinea pig plasma-matrix matched control (MMC) plasma. MMC plasma was spiked to 10 μg/mL with the oxime and processed as described in Methods. 2-PAM was eluted isocratically with 0.03 M ammonium acetate pH 4.5:44% acetonitrile, and TMB-4 was eluted with 0.21 M ammonium acetate:44% acetonitrile of 10 μL injections of 10μg/mL.

for 2-PAM and consistently 70% for TMB-4, except for the lowest concentration of 0.1μg/mL which yielded only 38% recovery. To maximize accuracy and account for variable recoveries, the standard curves were derived from matrix matched controls of AGM and GP. Equimolar amounts of the two salts of MMB-4 yielded the same HPLC retention time and peak absorbance, and the breakthrough fraction (plasma components which do not bind to the column) were observed within the first 2.5 min but the peak returned to baseline before the MMB-4 eluted (~3.5 minutes). The MMB-4 HPLC conditions were: 0.19 M ammonium acetate pH 4.5:44% acetonitrile and detection at 300 nm.

MMB-4 was stable for up to 8 hours with no detectable degradation at 4°C, which allowed processing of 96 samples in a refrigerated autosampler (data not

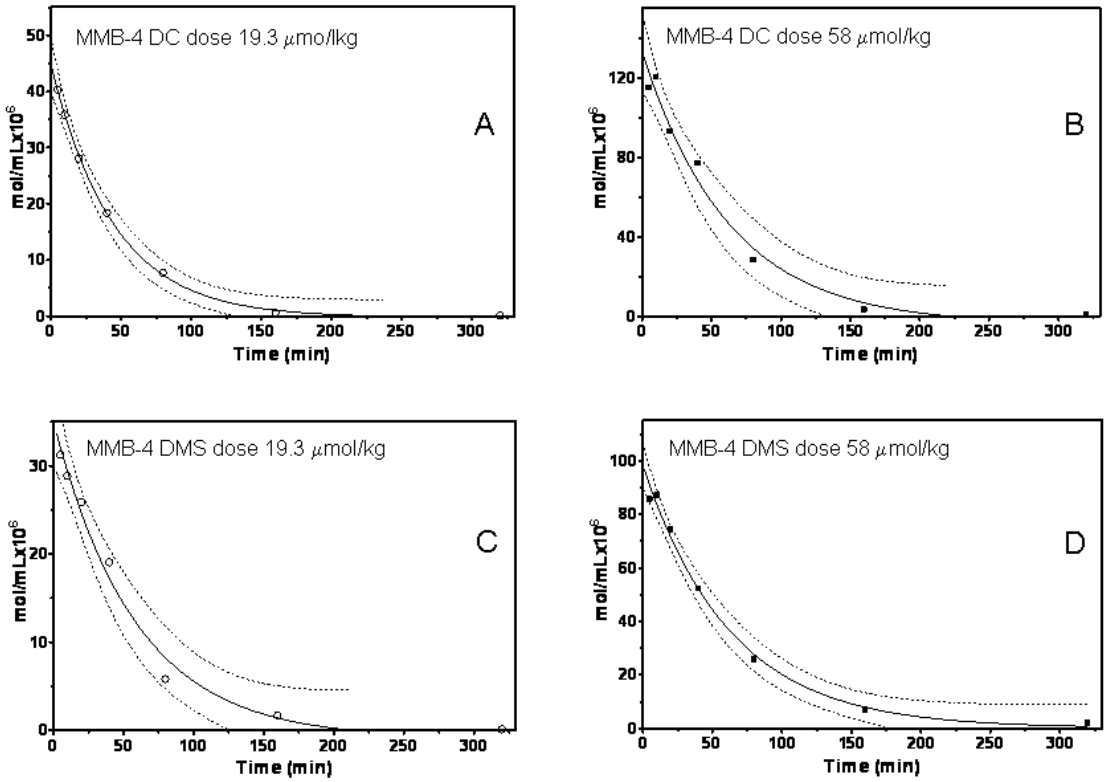

Fig. 3. Pharmacokinetic profile of MMB-4 in guinea pig plasma following a single i. m. dose. MMB-4 DC; C + D, MMB-4 DMS. Mean, CI:95%, n = 2 animal.

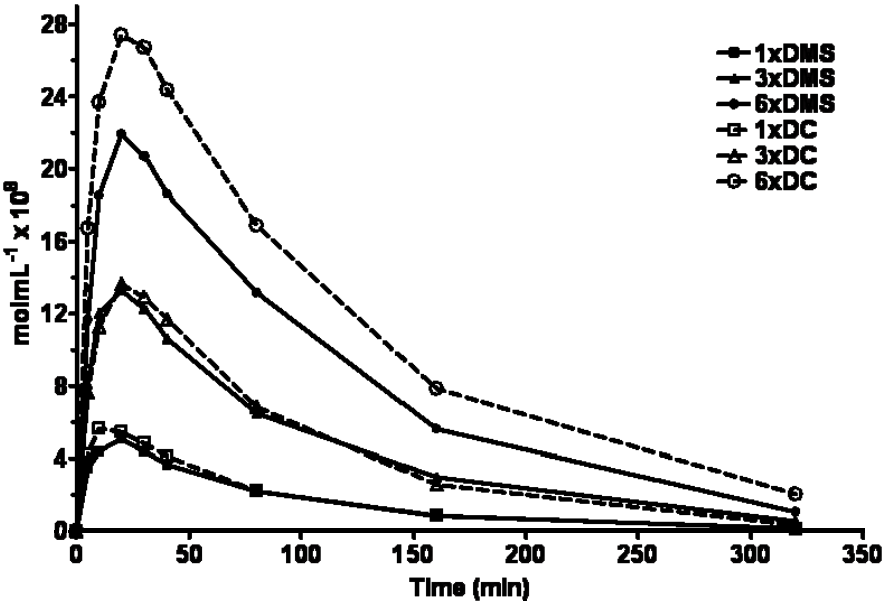

Fig. 4. Time-course of MMB-4 in plasma. Six curves are shown. As indicated in the legend, filled symbols represent DMS and empty symbols represent diCl. Squares, triangles, and circles represent 1X, 3X, and 6X doses, respectively.

Dose		19.3 umo//kg (1 autoInjector; 1X)				58 umo//kg (3 autoInjectors; 3X)				116 umo//kg (6 autoInjectors; 6X)			
				95% CI				95% CI				95% CI	
Salt		Mean	SD	lower	upper	Mean	SD	lower	upper	Mean	SD	lower	upper
DC	A_{rate}	0.17	0.08	0.11	0.24	0.09	0.04	0.06	0.12	0.11	0.06	0.05	0.16
	C_{max}	6.00	1.47	4.77	7.23	14.07	3.28	11.55	16.59	28.47	5.43	22.77	34.17
	T_{max}	16.25	7.44	10.03	22.47	22.22	8.33	15.82	28.63	20.00	10.95	8.50	31.50
	$T_{1/2}$	53.79	8.48	46.70	60.87	52.52	3.85	49.57	55.48	72.55	34.49	36.35	108.70
	MRT	77.88	8.71	70.59	85.16	84.70	9.72	77.23	92.17	113.10	47.64	63.14	163.10
	AUC	511.00	77.00	447.00	576.00	1358.00	314.00	1116.00	1599.00	3497.00	1519.00	1902.00	5091.00
DMS	A_{rate}	0.17	0.05	0.13	0.22	0.12	0.06	0.07	0.17	0.11	0.05	0.07	0.14
	C_{max}	5.18	0.69	4.60	5.76	13.73	2.19	12.05	15.42	22.16	3.70	19.07	25.25
	T_{max}	17.50	4.63	13.63	21.37	21.11	7.82	15.10	27.12	25.00	7.56	18.68	31.32
	$T_{1/2}$	65.63	12.64	55.06	76.19	63.62	5.72	59.23	68.02	64.46	4.79	60.03	68.88
	MRT	85.71	8.12	78.92	92.50	92.80	13.60	82.35	103.30	99.51	16.97	83.82	115.20
	AUC	455.00	87.00	382.00	528.00	1437.00	209.00	1277.00	1598.00	2566.00	299.00	2316.00	2817.00

Table 1. African green monkey pharmacokinetic descriptive statistical results for the 19.3 (1X), 58 (3X), and 116 (6X) umol/kg doses.

shown). Isocratic elution of 2-PAM occurred with 0.03 M while TMB-4 eluted with 0.21 M ammonium acetate. The GP or AGM plasma LOD for MMB-4 was 0.5 ng in 10 μL. The LOQ was 0.1 to 250 μg/mL with a 5-fold signal-to-noise ratio.

The intramuscular doses of MMB-4 in GP and AGM were chosen to reflect the amount of HI-6 used by the Swedish military; GP doses were 1 and 3 mg/kg human equivalent dose while the AGM doses were 1, 3, and 6 mg/kg human equivalent dose injection (0.05 μg/mL) The plasma LOD for MMB-4 was 0.5 ng in a 10 μL injection (0.05 μg/mL).

In GP, as shown in Fig. 3, both salts of MMB-4 yielded similar PK properties with a half-life ($T_{1/2}$) of 34 to 42 min, and the AUC increased dose-dependently. MMB-4 entered the plasma so rapidly that even the initial collection of blood 5 minutes after dosing was too late to determine the absorptive parameter.

For AGM, the pharmacokinetic profile is shown in Fig. 4. The derived pharmacokinetic properties are shown in Table I. C_{max} and AUC varied dose-dependently, the T_{max} was about 20 min and the mean residence time was 92 min for all doses of both salts, which fit a single compartment model. PK of MMB-4 in tissues was determined only for the GP.

GP tissue concentrations of MMB-4 were markedly lower than observed for plasma (data not shown). Depending on the tissue, MMB-4 levels peaked at 5-20 minutes: the rank order of concentration was diaphragm > heart > thigh muscle, and likely reflects differences in vascularization of the tissues, rapid breathing, and heart rate compared to the sedentary state of the animal.

In summary, we devised a general cation-exchange HPLC assay for mono- and di-pyridinium oximes in plasma and tissues. The assay is applicable to quantifying mono- and di-pyridinium oximes in plasma and tissues from rodents, non-human primates, and humans. The assay is reproducible, uses minimal processing, and requires only a small sample, is rapid, and sensitive. It is being developed under GLP procedures so that safety and efficacy testing in animals and safety evaluation in humans can be performed in support of FDA requirements. We found no difference in the PK of the two salts of MMB-4 in either the GP or AGM after a single i. m. dose. While the C_{max} and AUC increased dose-dependently in GP and AGM, the absorption and elimination of MMB-4 was more rapid in the GP than the AGM. Our HPLC assay provides the methods and initial data that the FDA requires to evaluate a new therapeutic oxime for OP poisoning that might result from chemical warfare agent or pesticide exposure to troops in the field, first responders, or the civilian population.

4. ACKNOWLEDGEMENT

This material has been reviewed by the Walter Reed Army Institute of Research and there is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense.

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